

Automated Dose-Response Analysis and Comparative Toxicogenomic Evaluation of the Hepatic Effects Elicited by TCDD, TCDF, and PCB126 in C57BL/6 Mice

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The toxic equivalency factor (TEF) approach recommended by the World Health Organization is used to quantify dioxin-like exposure concentrations for mixtures of polychlorinated dibenzo-dioxins, -furans, and polychlorinated biphenyls (PCBs), including 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 3,3',4,4',5-pentachlorobiphenyl (PCB126) relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Whole-genome microarrays were used to evaluate the hepatic gene expression potency of TCDF and PCB126 relative to TCDD with complementary histopathology, tissue level analysis, and ethoxyresorufin-*O*-deethylase (EROD) assay results. Immature ovariectomized C57BL/6 mice were gavaged with 0.001, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, and 300 μ g/kg TCDD and TEF-adjusted doses (TEF for TCDF and PCB126 is 0.1) of TCDF or PCB126 (1, 3, 10, 30, 100, 300, 1000, and 3000 μ g/kg of TCDF or PCB126) or sesame oil vehicle and sacrificed 24 h post dose. In general, TCDD, TCDF, and PCB126 tissue levels, as well as histopathological effects, were comparable when comparing TEF-adjusted doses. Automated dose-response modeling (ToxResponse Modeler) of the microarray data identified 210 TCDF and 40 PCB126 genes that exhibited sigmoidal dose-response curves with comparable slopes when compared with TCDD. These similar responses were used to calculate a median TCDF gene expression relative potency (REP) of 0.06 and a median PCB126 gene expression REP of 0.02. REPs of 0.02 were also calculated for EROD induction for both compounds. Collectively, these data suggest that differences in the ability of the liganded aryl hydrocarbon receptor:AhR nuclear translocator complex to elicit differential hepatic gene expression, in addition to pharmacokinetic differences between ligands, influence their potency in immature ovariectomized C57BL/6 mice.

Key Words: TCDD; TCDF; PCB126; TEF; mouse; dose-response.

Polychlorinated aromatic hydrocarbons (PAHs), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related chemicals, such as 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 3,3',4,4',5-pentachlorobiphenyl (PCB126), are ubiquitous environmental contaminants that elicit a broad spectrum of species-specific biochemical and toxic effects. Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are by-products of waste combustion, herbicide production, and other industrial processes (Commoner *et al.*, 1987; Mason and Safe, 1986; Poland and Glover, 1973; Safe *et al.*, 1982). In contrast, polychlorinated biphenyls (PCBs) were manufactured between 1930 and 1977 as mixtures that included PCB126 as well as other congeners (Mayes *et al.*, 1998). PCBs were used as coolants, lubricants, and dielectric insulating fluids for transformers and capacitors (Mullin *et al.*, 1984; Safe, 1990; Safe *et al.*, 1982). Even though their production has ceased, they continue to be released into the environment through the mishandling of PCB-containing products (National Toxicology Program [NTP], 2006b).

Although there are 75, 135, and 209 possible PCDD, PCDF, and PCB congeners, respectively, only 7, 10, and 12 are considered dioxin-like based on their ability to bind and activate the aryl hydrocarbon receptor (AhR) (Van den Berg *et al.*, 2006). The AhR is a cytosolic ligand-activated basic helix-loop-helix Per-Arnt-Sim domain containing transcription factor (Denison and Heath-Pagliuso, 1998; Denison *et al.*, 2002; Poland and Knutson, 1982; Safe, 2001). Following ligand binding, chaperone proteins that maintain the AhR in an inactive state dissociate from the AhR allowing translocation to the nucleus and heterodimerization with AhR nuclear translocator (ARNT). The AhR:ARNT heterodimer complex then interacts with dioxin response elements in the regulatory regions of target genes followed by recruitment of coregulatory

proteins, leading to changes in gene expression (Denison and Heath-Pagliuso, 1998; Nebert *et al.*, 2000).

Environmental exposure to PCDDs, PCDFs, and PCBs typically occurs as a complex mixture. In order to estimate the risk associated with a mixture, the concentration and potency of each toxic PCDD, PCDF, and PCB congeners are taken into account based on its toxic equivalency factor (TEF) relative to TCDD, the most toxic congener (Ahlborg, 1994; Barnes, 1991; Birnbaum and DeVito, 1995; Haws *et al.*, 2006; Van den Berg *et al.*, 1998, 2006). TEFs are single point potency estimates that were developed from relative potency (REP) values calculated by comparing the effective dose (ED₅₀) value of a single response elicited by TCDD and dividing it by the ED₅₀ for the same response elicited by the congener of interest (Van den Berg *et al.*, 1998, 2006). Consequently, the uncertainty of a TEF point estimate can extend over orders of magnitude, reflecting the range of REP values available for a particular congener in various model systems (Harris *et al.*, 1993; Haws *et al.*, 2006; Poland and Knutson, 1982; Safe, 1997, 1990; Starr *et al.*, 1999; Van den Berg *et al.*, 2006). TEFs are independent of dose, time point, and tissue and largely reflect biochemical effects such as enzyme induction. Although results from cancer bioassays or developmental-reproductive studies have been considered and are the critical human health risk assessment endpoints for PCDDs and PCDFs, no TEF value is based exclusively on these endpoints. Furthermore, it is assumed that at submaximal doses, the contribution of each congener is additive. There are also pharmacokinetic and distributional differences between congeners that may affect their REPs (Budinsky *et al.*, 2006; DeVito *et al.*, 1997, 1998; Diliberto *et al.*, 2001; Safe, 1995).

The TEFs for TCDF and PCB126 are currently set at 0.1, indicating that they are 10 times less potent than TCDD. However, our recent studies suggest that the TEF of 0.1 does not accurately reflect the hepatic potency of TCDF and PCB126 relative to TCDD in C57BL/6 mice (Burgoon *et al.*, 2009; Kopec *et al.*, 2008; N'Jai *et al.*, 2008). Although pharmacokinetic differences are an important factor, temporal and dose-dependent microarray data suggest that TCDF- and PCB126-elicited gene expression responses are also not equivalent, in terms of potency and efficacy, relative to TCDD at TEF-adjusted equivalent doses. To further investigate this hypothesis, parallel TCDD, TCDF, and PCB126 dose-response studies were conducted at 24 h to minimize the pharmacokinetic and distributional differences between these compounds. In addition to using the same species, comparable study designs and analysis methods were also used as previously reported (Burgoon *et al.*, 2009; Kopec *et al.*, 2008; N'Jai *et al.*, 2008). Moreover, complementary histopathology, tissue level analysis, and ethoxyresorufin-*O*-deethylase (EROD) activities were assessed. Results from this study not only expand the available REP data in C57BL/6 mice but also suggest that TCDF- and PCB126-elicited differential gene expression responses are not comparable to TCDD at TEF-adjusted equivalent doses at 24 h when pharmacokinetic and distributional differences are minimized.

TABLE 1
The 24 h Dose-Response Study Design

Dose ($\mu\text{g/kg}$)	Number of animals			Vehicle (sesame oil)
	TCDD	PCB126	TCDF	
0	—	—	—	7
0.001	4	—	—	—
0.01	4	—	—	—
0.03	4	—	—	—
0.1	4	—	—	—
0.3	4	—	—	—
1	4	4	4	—
3	4	4	4	—
10	4	4	4	—
30	4	4	4	—
100	4	4	4	—
300	4	4	4	—
1000	—	4	4	—
3000	—	4	4	—

MATERIALS AND METHODS

Animal husbandry. Female C57BL/6 mice, ovariectomized by the supplier on postnatal day (PND) 20, with body weights (BW) within 10% of the average, were obtained from Charles Rivers Laboratories (Kingston, NY) on PND 25. Animals were housed in polycarbonate cages containing cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensburg, NY) with 30–40% humidity and a 12-h light/dark cycle (0700 h–1900 h). Mice had free access to deionized water and Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI). Animals were dosed on PND 28 following acclimatization for 3 days. Immature mice were used because they are more responsive to AhR ligands and to facilitate comparisons with other data sets obtained using the same model, study design, and analysis methods (Boverhof *et al.*, 2005; Kopec *et al.*, 2008; N'Jai *et al.*, 2008). Animals were ovariectomized to negate potential interactions with estrogens produced by the developing ovaries because some animals are approaching reproductive maturity. All procedures were carried out with the approval of the Michigan State University Institutional Animal Care and Use Committee.

Dose-response study. A stock solution of PCB126 (99.7% purity; AccuStandard, New Haven, CT) was dissolved in acetone (J.T. Baker, Phillipsburg NJ), followed by dilution in sesame oil (Sigma, St. Louis, MO), and evaporation of the acetone using a mild stream of nitrogen gas. The PCB126 stock was further diluted in sesame oil to achieve the desired doses. TCDD and TCDF were gifts from The Dow Chemical Company (Midland, MI). Animals were orally gavaged using 1.5-inch feeding needle with a 2.25-mm ball end (Cadence Science, Lake Success, NY). Mice received 0.1 ml of a single dose of 0.001, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, and 300 $\mu\text{g/kg}$ of TCDD or 1, 3, 10, 30, 100, 300, 1000, and 3000 $\mu\text{g/kg}$ of PCB126 or TCDF or 0.1 ml sesame oil vehicle and sacrificed 24 h post dose. PCB126 and TCDF doses were TEF adjusted to be equivalent to the TCDD doses (Van den Berg *et al.*, 2006) (Table 1). Mice were sacrificed by cervical dislocation and tissue samples were removed, weighed, flash frozen in liquid nitrogen, and stored at -80°C . The right lobe of the liver was fixed in 10% neutral buffered formalin (Sigma) for histological analysis.

Histological analysis. Fixed liver tissues were sectioned and processed in ethanol, xylene, and paraffin using a Thermo Electron Excelsior tissue processor (Waltham, MA). Tissues were embedded in paraffin with Miles

Tissue Tek II embedding center after which paraffin blocks were sectioned at 5 μ m with a rotary microtome. Liver sections were placed on glass microscope slides, washed twice in xylene for 5 min, followed by four quick washes in ethanol, and rinsed in water. Slides were placed in Gill 2 hematoxylin (Thermo Fisher Scientific, Waltham, MA) for 1.5 min followed by two to three quick dips in 1% glacial acetic acid water and rinsed with running water for 2–3 min. Slides were then rinsed in ethanol and counterstained with 1% eosin Y-phloxine B solution (Sigma) followed by multiple rinses in ethanol and xylene. Coverslips were attached using aqueous mounting media. All the histological processing was performed at Michigan State University Investigative HistoPathology Laboratory, Division of Human Pathology, using a modified version of previously published procedures (Sheehan and Hrapchak, 1980).

Quantification of hepatic TCDD, PCB126, and TCDF levels. Liver samples were processed in parallel with laboratory blanks and a reference or background sample at Wellington Laboratories Inc. (Guelph, ON, Canada). The samples (100–500 mg) were transferred to a tared screw cap culture tube and weights were recorded. Samples were then spiked with a mixture of $^{13}\text{C}_{12}$ -2,3,7,8-TCDD, $^{13}\text{C}_{12}$ -PCB126, and $^{13}\text{C}_{12}$ -2,3,7,8-TCDF surrogates and digested with HCl. Each digest was split between two screw cap tubes and 3–4 ml hexane was added to each tube followed by vigorous mixing. The tubes were centrifuged, and the organic layer was removed. The hexane extraction was performed three times per tube with the six hexane fractions combined. The hexane fraction was then split and one fraction was archived. The other fraction was processed further using a small multilayer (acid/base/neutral) silica gel column eluted with 20–25 ml of hexane. The eluate was concentrated and transferred to a conical microvial with pentane and dichloromethane rinses and allowed to dry. Immediately prior to injection on the high-resolution gas chromatograph/high-resolution mass spectrometer (HRGC/HRMS) system, a mixture of $^{13}\text{C}_{12}$ -1,2,3,4-TCDD, $^{13}\text{C}_{12}$ -PCB111, and $^{13}\text{C}_{12}$ -1,2,3,4-TCDF injection standards were added to the conical microvial. TCDD, PCB126, and TCDF analyses were performed using an Agilent (Agilent Technologies, Inc., Santa Clara, CA) 6890 series HRGC with direct capillary interface to a Waters (Milford, MA) Autospec Ultima HRMS. Chromatographic separations were carried out on a 60-m DB5 (0.25 mm internal diameter and 0.25 μ m film thickness) column in constant flow mode (Helium, 1 ml/min). All injections were 1 μ l using splitless injection. The mass spectrometer was operated in positive electron ionization selective ion recording mode at a mass resolving power of 10,000 or greater.

EROD assay. Microsomes were extracted from ~100 mg samples by differential centrifugation (Moore *et al.*, 2009). Tissue was minced and homogenized using Tri-R Stir-R homogenizer (Tri-R Instruments, Inc., Rockville Centre, NY) in 0.05M Tris (Invitrogen, Carlsbad, CA), 1.15% KCl (J.T. Baker), pH 7.5, and then centrifuged at 4°C, 10,000 \times g for 10 min. The supernatant was centrifuged at 4°C, 100,000 \times g for 30 min. The microsomal pellets were resuspended in 0.01M EDTA (Invitrogen), and 1.15% KCl, pH 7.4, and recentrifuged at 100,000 \times g for 60 min. Final pellets were resuspended in a stabilizing buffer (20% glycerol [J.T. Baker], 0.1M KH_2PO_4 [J.T. Baker], and 1mM EDTA and 1mM dithiothreitol [Roche Applied Science, Indianapolis IN], pH 7.25) and stored at –80°C. Extracted hepatic microsomes from vehicle, TCDD-, PCB126-, and TCDF-treated mice were assayed for EROD activity by monitoring the production of resorufin measured at 590 nm in a 96-well plate (Costar, Corning, NY) using a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific) and a corresponding software (version 2.6). The assay was performed in 0.05M HEPES (Sigma), pH 7.8, and 3.35mM 7-ethoxyresorufin (Molecular Probes, Eugene OR). Catalytic activity was initiated by addition of 0.3mM NADPH (Sigma), and fluorescence was measured every 2 min. After 30 min, the assay was terminated by addition of 36 μ g fluorescamine (Sigma) in acetonitrile. Protein concentrations were measured at 460 nm using bovine serum albumin (Roche Applied Science) as a protein standard. Linear portions of each kinetic profile analysis were used to determine picomoles of resorufin produced per minute and standardized to the total protein (mg).

RNA isolation. Frozen liver samples (left lobe, ~100 mg stored at –80°C) were immediately transferred to 1 ml TRIzol (Invitrogen) and homogenized

using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was isolated according to the manufacturer's protocol with an additional acid phenol:chloroform extraction. Isolated RNA was resuspended in RNA storage solution (Ambion Inc., Austin, TX) and quantified (A_{260}). RNA quality was assessed by determining the A_{260} : A_{280} ratio and visual inspection of 2 μ g on a denaturing gel.

Microarray experimental design. Treated and vehicle RNA samples were individually hybridized to 4 \times 44 K Agilent oligonucleotide microarrays (Agilent Technologies, Inc.). Three biological replicates were performed using one-color labeling (Cy3) for each dose according to the manufacturer's protocol (Agilent Manual: G4140-90040 v. 5.7). Microarray slides were scanned at 532 nm (Cy3) on a GenePix 4000B scanner (Molecular Devices, Union City, CA). Images were analyzed for feature and background intensities using GenePix Pro 6.0 (Molecular Devices). All data were archived in TIMS dbZach data management system (Burgoon and Zacharewski, 2007).

Microarray analysis. All microarray data in this study passed our laboratory quality assurance protocol (Burgoon *et al.*, 2005). TCDD, PCB126, and TCDF data sets were independently normalized because of the overall size of the files using a semiparametric approach (Eckel *et al.*, 2005). Posterior probabilities were then calculated using an empirical Bayes method on a per-gene and dose basis using model-based *t* values (Eckel *et al.*, 2004). Gene expression data were then ranked and prioritized ($P_1(t)$ values > 0.99 or > 0.90 and |fold change| > 1.5) to identify differentially expressed genes. Complete dose-response microarray data sets are available as Supplementary tables 3–5.

Quantitative real-time PCR. Quantitative real-time PCR (QRT-PCR) was performed as previously described (Boverhof *et al.*, 2005). Briefly, 1 μ g of total RNA was reverse transcribed by SuperScript II (Invitrogen) using an anchored oligo-dT primer. The complementary DNA (cDNA) (1 μ l) was used as a template in a 30- μ l reaction containing 0.1 μ M of forward and reverse gene-specific primers, 3mM MgCl_2 , 1mM dNTPs, 0.025 IU AmpliTaq Gold, and 1X SYBR Green PCR buffer (Applied Biosystems, Foster City, CA). Supplementary table 2 provides the names, abbreviations, accession numbers, forward and reverse primer sequences, and amplicon sizes. Amplification was conducted on an Applied Biosystems PRISM 7500 Sequence Detection System. The cDNAs were quantified using a standard curve approach, and the copy number of each sample was standardized to three housekeeping genes (Actb, Gapdh, and Hprt) (Vandesompele *et al.*, 2002). For graphing purposes (GraphPad Prism 5.0), the relative expression levels were scaled such that the expression level of the time-matched control group was equal to one.

Dose-response modeling. Dose-response modeling was performed using the ToxResponse Modeler (Burgoon and Zacharewski, 2008). ToxResponse Modeler performs automated dose-response modeling by identifying the best-fit model among five different mathematical models (linear, exponential, Gaussian, sigmoidal, and quadratic). The algorithm then chooses the best-fit model of the five best in-class models. The overall best-fit model is then used to calculate the ED_{50} values. Microarray dose-response data were first filtered using a $P_1(t)$ > 0.90 cutoff and filtered to identify genes exhibiting a sigmoidal dose-response profile. Supplementary table 6 contains the list of sigmoidal dose-responsive genes together with corresponding ED_{50} and REP values. REP values were calculated on a per-feature/per-gene basis using model-based ED_{50} values:

$$\text{REP} = \frac{\text{TCDD } \text{ED}_{50}}{\text{TCDF or PCB126 } \text{ED}_{50}}.$$

Statistical analysis. All statistical analyses were performed with SAS 9.1 (SAS Institute, Cary, NC). All data (with the exception of microarray data) were analyzed by ANOVA followed by Dunnett's or Tukey's *post hoc* tests. Differences between treatment groups were considered significant when $p < 0.05$.

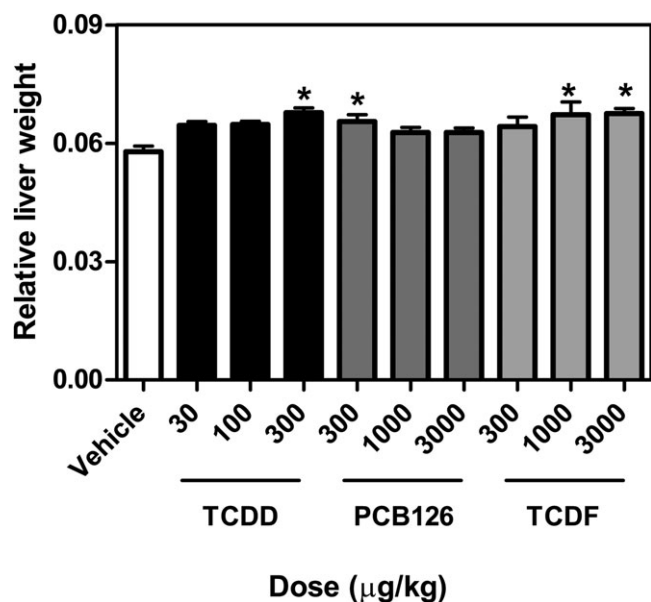


FIG. 1. Dose-dependent changes in the RLWs 24 h post dose for TCDD, PCB126, and TCDF. Results are displayed as mean \pm SE of at least four independent replicates. Data were analyzed by ANOVA followed by Dunnett's *post hoc* test: * $p < 0.05$ for vehicle versus treated samples. No additional significant treatment-related alterations in liver or organ weights were noted in the dose-response study.

RESULTS

Liver and BWs

Rodents exposed to PCAHs including dioxins, PCBs, and furans exhibit temporal and dose-dependent hepatic changes characterized by increases in liver weight (Denison and Heath-Pagliuso, 1998; NTP, 2006a,b). PCB126 and TCDF have TEFs of 0.1 (Van den Berg *et al.*, 2006), indicating that 10 times more chemical is required to elicit similar effects compared with an equivalent dose of TCDD. In our study, PCB126 and TCDF doses were TEF adjusted to theoretically match the potency of TCDD. Significant ($p < 0.05$) increases in relative liver weight (RLW) were observed with 300 µg/kg TCDD, 300 µg/kg PCB126, and 1000 and 3000 µg/kg TCDF in agreement with previous reports using the same compounds, animal models, and study designs (Fig. 1) (Boverhof *et al.*, 2005, 2006; Kopec *et al.*, 2008; N'Jai *et al.*, 2008). No changes in BW or BW gain relative to vehicle controls were observed within 24 h, indicating no systemic toxicity or wasting syndrome response at the doses used.

Hepatic TCDD, PCB126, and TCDF Levels

TCDD, PCB126, and TCDF levels per liver wet weight (in pg/g) were measured in three individual samples per dose using HRGC/HRMS. A dose-dependent increase in the hepatic concentration of all three compounds was observed (Figs. 2A–C and Table 2). The tissue levels of TCDD, PCB126, and TCDF were significantly ($p < 0.05$) higher compared to vehicle

controls, except for the lowest doses of TCDD (0.001–0.1 µg/kg), because of the presence of low background levels of TCDD. At TEF-equivalent doses, the levels of all three compounds were comparable except for statistical differences at 0.1, 0.3, 100, and 300 µg/kg (Supplementary fig. 1). The use of TEF values for determining tissue concentrations increases the uncertainty and reliability of potency estimates (Van den Berg *et al.*, 2006). Despite this limitation, the use of TEF-adjusted tissue level data suggests that the potencies of TCDD, TCDF, and PCB126 should be comparable because there were minimal differences in hepatic absorption, distribution, and metabolism between compounds at 24 h (Supplementary fig. 1). This is significant because pharmacokinetic differences between these ligands have been reported to contribute to substantial differences in potencies at later time points (Budinsky *et al.*, 2008; DeVito and Birnbaum, 1995; Kopec *et al.*, 2008; N'Jai *et al.*, 2008).

Histopathology

Hematoxylin and eosin staining was examined on the three highest TEF-equivalent doses (30, 100, and 300 µg/kg TCDD; 300, 1000, and 3000 µg/kg PCB126 and TCDF). TCDD, PCB126, and TCDF elicited dose-dependent increases in periportal hepatocellular vacuolization and multifocal mixed inflammatory infiltration (Table 3 and Fig. 3). TCDD and the equivalent TEF-matched TCDF doses elicited comparable hepatic vacuolization, whereas TEF-equivalent PCB126 doses exhibited slightly less vacuolization (Table 3). Hepatocellular single cell necrosis was present only in the highest dose groups for all three compounds with the most pronounced necrosis occurring with 3000 µg/kg TCDF (Table 3 and Fig. 3). Comparable levels of dose-dependent mixed inflammatory cell foci (lymphocytes, monocytes, and neutrophils) were observed. Qualitatively, these results suggest that TEF-matched doses of TCDD, PCB126, and TCDF elicit comparable histopathological effects at 24 h. Supplementary table 1 contains a detailed report on the incidence and severity of histopathological responses.

EROD Activity

Hepatic EROD activity was assessed to anchor our results to data within the World Health Organization (WHO) REP database (Haws *et al.*, 2006). TCDD induced a dose-dependent sigmoidal increase in EROD activity with an ED₅₀ of 1.1 µg/kg, calculated using the ToxResponse Modeler (Burgoon and Zacharewski, 2008) (Fig. 4). TEF-matched doses of PCB126 and TCDF elicited comparable EROD induction (Fig. 4) but were less potent than TCDD (PCB126 ED₅₀ = 53.6 µg/kg and TCDF ED₅₀ = 54.2 µg/kg). These EROD activity REPs (0.02 for PCB126 and TCDF) are consistent with values reported in the WHO REP database (Haws *et al.*, 2006). Moreover, in our published study examining TEF-equivalent doses of TCDD and TCDF at 72 h, the ToxResponse Modeler identified

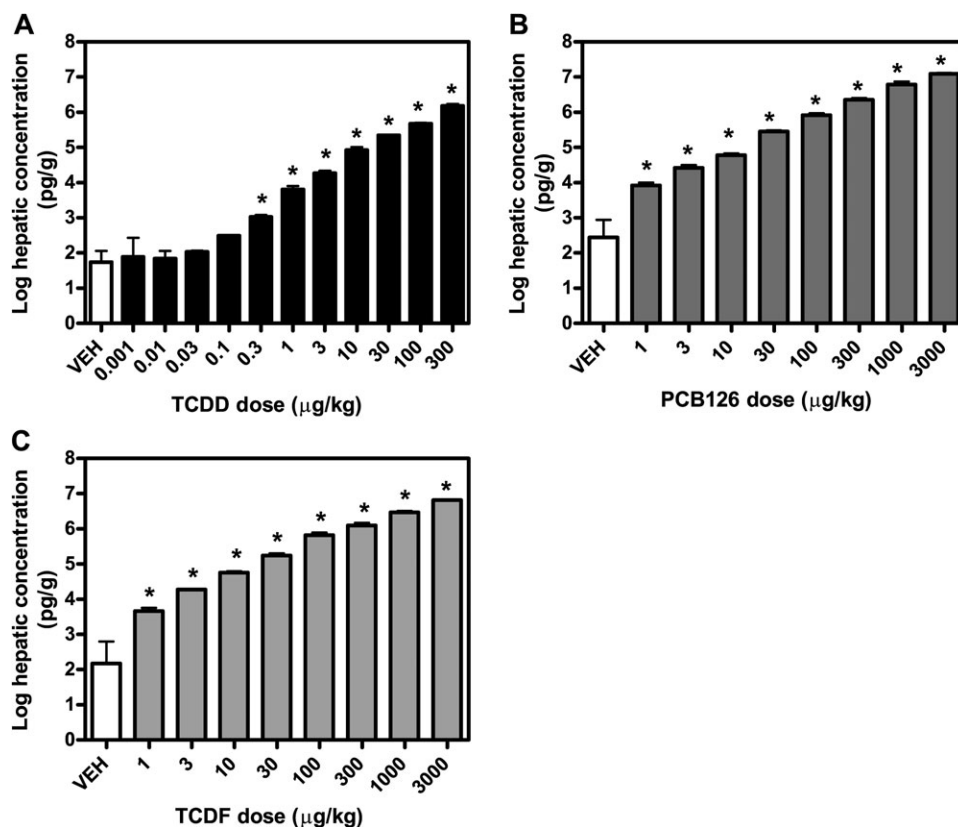


FIG. 2. Hepatic (A) TCDD, (B) PCB126, and (C) TCDF levels per g liver wet weight measured 24 h post dose using HRGC/HRMS. The data are displayed on a log scale to visualize tissue concentrations at all doses. The results are displayed as mean \pm SE of at least three independent samples. Data were analyzed by ANOVA followed by Dunnett's *post hoc* test: * $p < 0.05$ for vehicle versus treated samples.

a comparable EROD REP value (TCDF EROD REP_{72h} of 0.04) (Burgoon *et al.*, 2009).

Hepatic TCDF and PCB126 Gene Expression REPs

TCDF and PCB126 microarray data were analyzed to determine both per-gene and aggregate gene expression REP values using set theory and our ToxResponse Modeler (Figs. 5A–C). The analysis abides by assumptions underlying the TEF concept (e.g., same mechanism of action, parallel dose-response curves). TCDD, TCDF, and PCB126 microarray data sets were individually normalized (Eckel *et al.*, 2005) and analyzed using an empirical Bayes approach to identify differentially expressed features. Compared with previously published microarray studies (Boverhof *et al.*, 2005; Kopec *et al.*, 2008; N'Jai *et al.*, 2008), a more relaxed $P1(t)$ cutoff was used ($P1(t) > 0.90$) to be more inclusive, yielding higher statistical power at the risk of including more false positives at this early stage in the dose-response analysis.

TCDD elicited ~ 1.6 times more differentially expressed features when compared to TCDF (13,476 vs. 8655) (Fig. 5A) at comparable hepatic levels (Supplementary fig. 1). This trend is similar to the 72 h dose-response analysis, where TCDD elicited the differential expression of almost twice as

many features compared with TCDF, but at lower hepatic TCDF levels because of ligand-specific pharmacokinetics (Budinsky *et al.*, 2008; Burgoon *et al.*, 2009; DeVito and Birnbaum, 1995; Diliberto *et al.*, 1995, 2001; Hamm *et al.*, 2003). Likewise, TCDD differentially expressed ~ 1.3 times more features compared to PCB126 (13,476 vs. 10,983) (Fig. 5A). It should be noted that TCDD consistently elicited the greatest number of differentially expressed features (and genes in subsequent analysis) compared to either TCDF or PCB126.

Unions of the TCDD plus TCDF (16,227 features) and TCDD plus PCB126 (17,053 features) data sets were taken to include all differentially regulated features for further dose-response and REP analysis. Features missing data at one or more doses as well as the Agilent included controls were removed and not considered further. Features were then mapped back to genes to investigate dose-dependent changes in expression (Fig. 5A).

Genes with a $P1(t) > 0.90$ were also filtered for a >1.5 -fold change in expression for at least one dose relative to time-matched controls. This resulted in 5191 TCDD and 3919 TCDF genes that exhibited an expression change greater than 1.5-fold for at least one dose with a corresponding $P1(t) > 0.90$ and an ED₅₀ value within the experimental dose range (Fig. 5B).

TABLE 2
Absolute Hepatic Tissue Levels (in pg/g or ppt per wet weight) for TCDD-, PCB126-, and TCDF-Treated Samples and Vehicle Controls Measured by HRGC/HRMS

Dose (µg/kg)	Absolute hepatic tissue levels (in pg/g or ppt per wet weight)		
	TCDD	PCB126	TCDF
Vehicle (sesame oil)	$1.00 \times 10^2 \pm 9.84 \times 10^1$	$1.22 \times 10^3 \pm 1.98 \times 10^3$	$1.05 \times 10^3 \pm 1.68 \times 10^3$
0.001	$2.07 \times 10^2 \pm 2.39 \times 10^2$	—	—
0.01	$8.83 \times 10^1 \pm 7.12 \times 10^1$	—	—
0.03	$1.09 \times 10^2 \pm 1.29 \times 10^1$	—	—
0.1	$3.10 \times 10^2 \pm 2.06 \times 10^1$	—	—
0.3	$1.08 \times 10^3 \pm 2.21 \times 10^2$	—	—
1	$6.73 \times 10^3 \pm 2.21 \times 10^3$	$8.66 \times 10^3 \pm 2.58 \times 10^3$	$4.79 \times 10^3 \pm 1.56 \times 10^3$
3	$1.90 \times 10^4 \pm 4.85 \times 10^3$	$2.72 \times 10^4 \pm 9.24 \times 10^3$	$1.88 \times 10^4 \pm 1.70 \times 10^3$
10	$8.70 \times 10^4 \pm 2.78 \times 10^4$	$6.08 \times 10^4 \pm 1.23 \times 10^4$	$5.81 \times 10^4 \pm 6.36 \times 10^3$
30	$2.25 \times 10^5 \pm 1.06 \times 10^4$	$2.89 \times 10^5 \pm 2.72 \times 10^4$	$1.80 \times 10^5 \pm 3.81 \times 10^4$
100	$4.75 \times 10^5 \pm 5.08 \times 10^4$	$8.39 \times 10^5 \pm 2.06 \times 10^5$	$6.81 \times 10^5 \pm 2.24 \times 10^5$
300	$1.52 \times 10^6 \pm 3.07 \times 10^5$	$2.29 \times 10^6 \pm 4.92 \times 10^5$	$1.29 \times 10^6 \pm 3.52 \times 10^5$
1000	—	$6.41 \times 10^6 \pm 1.95 \times 10^6$	$2.96 \times 10^6 \pm 4.66 \times 10^5$
3000	—	$1.25 \times 10^7 \pm 1.01 \times 10^6$	$6.63 \times 10^6 \pm 4.05 \times 10^5$

Note. The values represent mean \pm SD of at least three independent replicates.

This means that of the 5191 genes differentially expressed by TCDD for at least one dose, 3120 genes were not represented in the TCDF data set and of the 3919 TCDF genes, 1848 were not represented in the TCDD data set (Fig. 5B). Compared with the 72 h dose-response analysis (Burgoon *et al.*, 2009), approximately five times more TCDD and TCDF genes were identified at 24 h (5191 TCDD and 3919 TCDF genes at 24 h compared with 1027 TCDD and 837 TCDF genes at 72 h).

The ToxResponse Modeler then identified the best linear, exponential, Gaussian, sigmoidal, and quadratic models for

each dose-responsive differentially expressed gene (e.g., best in class). The model that best fit the data among the different classes (e.g., best in show) was then used to calculate an ED₅₀ for each dose-responsive gene. Only those genes with an ED₅₀ within the experimental dose-response range were retained for subsequent REP analysis. The intersection of 5191 TCDD and 3919 TCDF genes identified 2071 genes that were used to calculate gene-specific REPs. Furthermore, 3120 and 1848 genes were TCDD- and TCDF specific, respectively. Of those, 2137 TCDD and 1630 TCDF genes exhibited sigmoidal dose-response curves. The intersection of 2071 genes in common

TABLE 3
Dose-Dependent Incidence and Severity of Hepatic Histopathological Responses in the Vehicle, TCDD-, PCB126-, and TCDF-Treated Mice at 24 h

	Treatment and dose (µg/kg)									
	Vehicle	TCDD			PCB126			TCDF		
		0	30	100	300	300	1000	3000	300	1000
Hepatocellular vacuolization										
Average severity	0	1	1.75	2.5	1	1.5	2	1.25	1.75	2.5
Hepatocellular single cell necrosis										
Average severity	0	0	0	0.5	0	0	0.5	0	0	1.25
Mixed cell infiltration										
Average severity	0	0.25	0.25	0.5	0	0.5	0.5	0	0.25	0.75

Note. The average severity scores are reported as a weighted average based on the following scoring scheme: minimal (grade of 1), slight (grade of 2), moderate (grade of 3), and/or marked (grade of 4) responses divided by the total number of examined animals. A comprehensive histopathological report with detailed incidence and severity is available in Supplementary table 1.

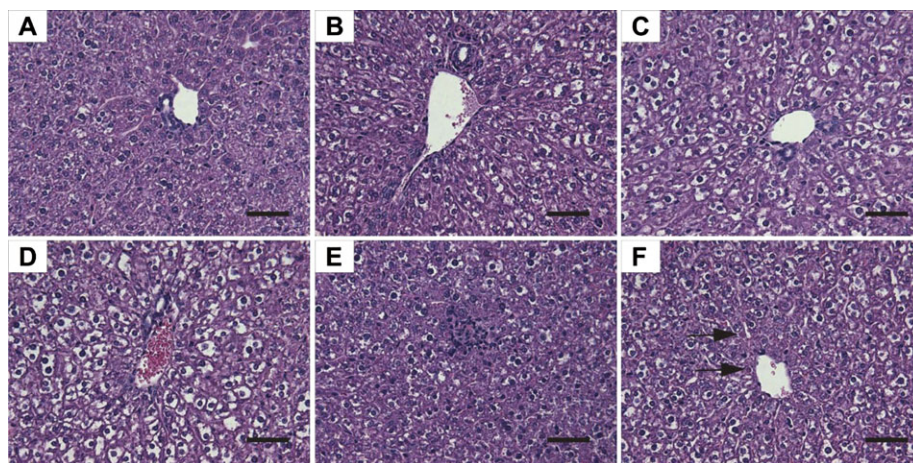


FIG. 3. Representative hematoxylin and eosin-stained liver sections 224 h postexposure to PCB126, TCDF, or TCDD. Selected doses resulted in vacuolization, single cell necrosis, and/or immune cell infiltration. (A) Vehicle treatment resulted in no visible hepatic alterations. (B) 300 µg/kg PCB126 elicited minimal vacuolization compared with (C) 1000 µg/kg TCDF and (D) 300 µg/kg TCDD, which exhibited more pronounced vacuolization. (E) 3000 µg/kg TCDF resulted in immune cell infiltration as well as (F) instances of necrosis (arrow). Bars = 50 µm.

between TCDD and TCDF with reasonable ED_{50} values identified 1506 genes with sigmoidal dose-response curves (Fig. 5B).

In order to comply with WHO TEF assumptions regarding similar slopes and curve shapes, only those genes that exhibited correlation coefficient greater than 60% were considered further for gene-specific REP analysis. In this study, the coefficient was lowered compared with the 72 h TCDF dose-response analysis (correlation coefficient > 80%) (Burgoon *et al.*, 2009) to include more genes and to account for the smaller overlap between TCDD and PCB126 data sets, thus facilitating comparisons between PCB126 and TCDF at 24 h. Correlation analysis identified 210 genes with similar slopes and curve shapes elicited by TCDD and TCDF (Fig. 5C). Sixteen of these genes had gene-specific REP values between

0 and 0.01, 28 between 0.01 and 0.03, 89 between 0.03 and 0.1, 51 between 0.1 and 0.3, 16 between 0.3 and 1, and 10 genes had REPs greater than 10. The median REP for all 210 genes was 0.06 lower than the 72 h median REP of 0.1 (0.096) calculated using 83 genes with similar slopes and curves for TCDD and TCDF (Burgoon *et al.*, 2009). Relaxing the correlation coefficient used for the 72 h dose-response study (Burgoon *et al.*, 2009) to 60% resulted in an almost identical median gene expression REP of 0.1 (0.112).

An identical approach was used to determine PCB126 gene-specific REPs and a median REP (Fig. 5C). In summary, only 40 genes exhibited similar slope and sigmoidal curves, of which 14 genes had REPs between 0 and 0.01, 12 between 0.01 and 0.03, 11 between 0.03 and 0.1, 2 between 0.3 and 1, and only 1 gene had a REP greater than 1. The median PCB126 gene expression REP for the 40 sigmoidal genes was 0.02. The PCB126 data set lost a majority of genes from further consideration because the ED_{50} s were not within the experimental dose range. The low number of dose-responsive genes considered for the PCB126 REP analysis can be attributed to the lower number of differentially expressed genes with sufficient potency when compared with TCDF and TCDD.

REPs based on tissue levels were also calculated for a subset of responsive genes used to determine REPs based on administered dose (Supplementary table 6). However, a vast majority of tissue level-based REPs could not be calculated because of differences in curve shapes and slopes. Overall, the median tissue level gene expression REPs for TCDF and PCB126 were comparable with administered dose-based REPs for the limited number of genes considered (TCDF $REP_{administered\ dose} = 0.06$, TCDF $REP_{tissue\ level} = 0.04$, and PCB126 $REP_{administered\ dose}$ and $REP_{tissue\ level} = 0.02$). Note that approximately 60% of the tissue level-based REPs were within an order of magnitude of the administered dose-based REPs.

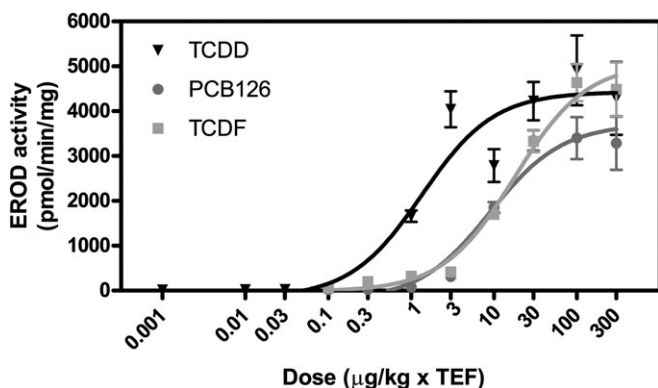


FIG. 4. Dose-dependent induction of EROD activity following exposure to TEF-equivalent doses of TCDD, PCB126, and TCDF. The ToxResponse Modeler calculated ED_{50} values of 1.1, 53.6, and 54.2 µg/kg for TCDD, PCB126, and TCDF, respectively, yielding PCB126 and TCDF EROD REPs of 0.02. Symbols represent the mean \pm SE of four independent samples. Sigmoidal dose-response curve fitting was done using GraphPad Prism 5.0.

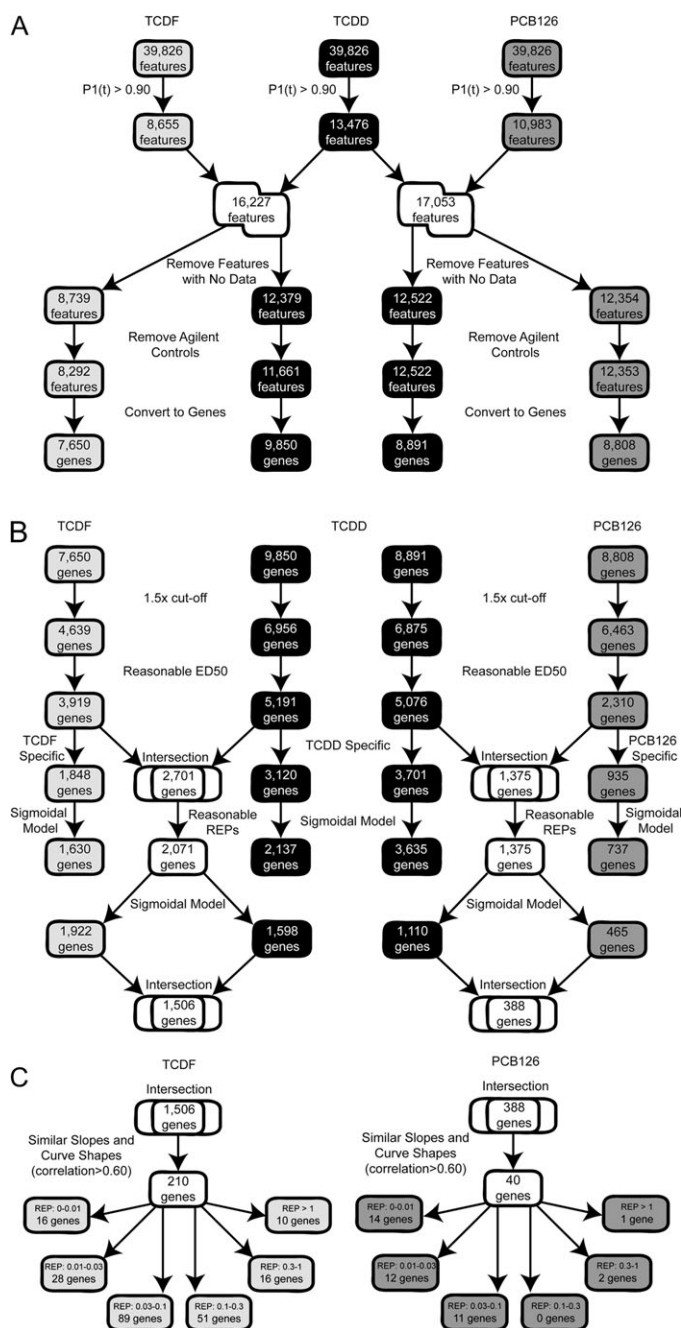


FIG. 5. (A) Comprehensive TCDD, TCDF, and PCB126 dose-response analysis—Part 1/3. Differentially regulated features were identified using a relaxed $P1(t)$ cutoff of 0.90 to maximize the number of differentially expressed genes under consideration. TCDD elicited ~1.6 times more differentially expressed features compared with TCDF (13,476 vs. 8,655) and ~1.3 times more differentially expressed features compared with PCB126 (13,476 vs. 10,983). Unions were taken to identify all differentially regulated features. Features missing data at any dose, as well as Agilent controls, were removed and not considered further. Features were mapped to specific genes for further analysis. (B) Comprehensive TCDD, TCDF, and PCB126 dose-response analysis—Part 2/3. Differentially expressed genes were analyzed further if the change in expression was greater than 1.5-fold for at least one dose. Genes exhibiting a sigmoidal dose-response were identified and intersected to identify genes responsive to both TCDD and TCDF and to

Microarray analysis suggests that there were a number of TCDF- and PCB126-specific responses. Three-way Venn analysis of the 3280, 2343, and 1411 gene expression changes elicited by TCDD, TCDF, and PCB126 ($P1(t) > 0.99$ and $|\text{fold change}| > 1.5$), respectively, identified only 202 genes differentially expressed by all three compounds (Fig. 6A). However, after relaxing the statistical cutoff ($P1(t) > 0.90$ and $|\text{fold change}| > 1.5$) (Fig. 6B), almost all PCB126- and TCDF-elicited gene expression changes overlapped, consistent with the conserved AhR-mediated mechanism of action. Overlaps between TCDD versus TCDF and TCDD versus PCB126 also significantly increased. The high number of TCDD-specific genes could be attributed to a wider range of doses used in the study (11 doses of TCDD vs. 8 doses of PCB126 and TCDF), and the greater potency and efficacy of TCDD even at TEF-adjusted doses of TCDF and PCB126. These results are in agreement with reports suggesting that TCDF- and PCB126-elicited responses are a subset of TCDD-regulated genes and consistent with a common AhR-mediated mode of action (Kopeck *et al.*, 2008; N'Jai *et al.*, 2008). At later time points, TCDD elicits more pronounced histopathological responses and changes in serum clinical chemistry compared with TCDF and PCB126. A more thorough discussion of the association between differential gene expression and pathology has been previously reported (Boverhof *et al.*, 2005, 2006; Kopeck *et al.*, 2008; N'Jai *et al.*, 2008). QRT-PCR confirmed the dose-dependent changes in expression for a subset of AhR-regulated genes (Fig. 7). The classic members of the “AhR gene battery” involved in phase I and phase II metabolism including Cyp1a1, Cyp1a2, and Nqo1 (Nebert *et al.*, 1990) were induced. In addition, Tiparp previously reported to exhibit robust AhR-dependent expression (Ma *et al.*, 2001; Tijet *et al.*, 2006) and Notch1 implicated in cell differentiation and suggested to have a role in normal AhR signaling during liver development (Boverhof *et al.*, 2005; Harper *et al.*, 2003) were also

TCDD and PCB126 at 24 h. Genes were examined further if the model-based ED_{50} value, calculated by ToxResponse Modeler, was within the experimental dose range. Genes exhibiting a sigmoidal dose-response curve for both TCDD and TCDF were intersected to identify 1506 genes that exhibited an expression change greater than 1.5-fold for at least one dose, a $P1(t) > 0.90$, a sigmoidal profile, and an ED_{50} value within the experimental range. Identical analysis yielded only 388 sigmoidal genes for TCDD and PCB126. PCB126 lost a majority of differentially expressed dose-responsive genes from further consideration because the ED_{50} s were not within the experimental dose range. (C) Comprehensive TCDD, TCDF, and PCB126 dose-response analysis—Part 3/3. In the final analysis, assumptions regarding similarities in the slopes and shapes of corresponding TCDD and TCDF as well as TCDD and PCB126 sigmoidal dose-response curves were assessed by calculating the correlation coefficients of the elicited dose-response curves. The correlation analysis identified 210 genes from TCDD versus TCDF comparisons, and only 40 genes from TCDD and PCB126 comparisons with correlation coefficients greater than 0.60. The distribution of individual gene expression REPs is provided. The median REP for hepatic gene expression in the immature ovariectomized C57BL/6 mouse at 24 h was calculated to be 0.06 and 0.02 for TCDF and PCB126, respectively.

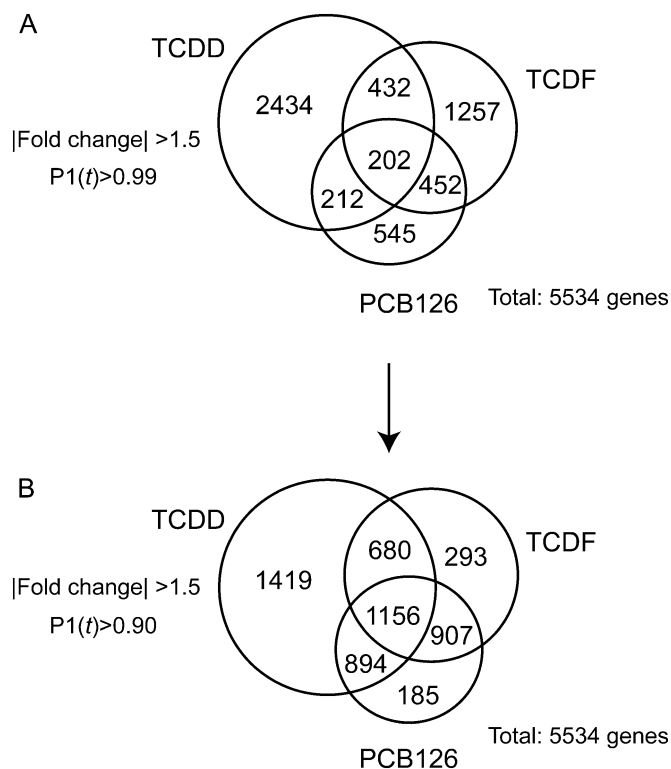


FIG. 6. Three-way Venn analysis of TCDD, PCB126, and TCDF elicited differentially expressed genes at (A) stringent ($|\text{fold change}| > 1.5$, $P1(t) > 0.99$) and (B) relaxed statistical criteria ($|\text{fold change}| > 1.5$, $P1(t) > 0.90$).

significantly expressed. The results from QRT-PCR analysis were in good agreement with the microarray data.

DISCUSSION

In this study, the dose-dependent hepatic gene expression effects of TEF-adjusted equipotent doses of TCDF and PCB126 (WHO TEF = 0.1) relative to TCDD (WHO TEF = 1) were examined at 24 h. In addition, complementary histopathology and EROD activity were assessed in order to associate dose-dependent changes in gene expression to higher order effects at comparable TEF-adjusted TCDD, TCDF, and PCB126 tissue levels. Unlike comparable studies at later time points, the TEF-adjusted tissue levels for TCDD, TCDF, and PCB126 were comparable at 24 h except for slight statistical differences at 0.1, 0.3, 100, and 300 $\mu\text{g/kg}$ (dose \times TEF). Therefore, differences in pharmacokinetics cannot be used to explain the weaker responses elicited by equipotent TEF-adjusted doses of TCDF and PCB126. By minimizing the differences in TEF-adjusted levels of TCDD, TCDF, and PCB126 in the liver, we further investigated qualitative and quantitative gene expression differences that may contribute to the weaker responses elicited by TCDF and PCB126.

Overall, the results of this 24 h study are consistent with our previous 72 h studies using a similar model, the same

oligonucleotide microarrays, and the same analysis methods (Burgoon *et al.*, 2009; Kopec *et al.*, 2008; N'Jai *et al.*, 2008). At the highest doses, 3000 $\mu\text{g/kg}$ TCDF, 3000 $\mu\text{g/kg}$ PCB126, and 300 $\mu\text{g/kg}$ TCDD elicited comparable increases in RLW accompanied by similar levels of hepatocellular vacuolization and minimal evidence of necrosis and immune cell infiltration at 24 h. Consequently, TEF-adjusted equipotent doses of TCDF and PCB126 elicited comparable phenotypic level effects when compared with TCDD. However, at later time points (72 and 168 h), with more apparent differences in tissue levels, TCDD, TCDF, and PCB126 elicited different levels of vacuolization, necrosis, and immune cell infiltration (Boverhof *et al.*, 2005; Burgoon *et al.*, 2009; Kopec *et al.*, 2008; N'Jai *et al.*, 2008), consistent with reported ligand-specific pharmacokinetics (DeVito *et al.*, 1998).

Despite comparable levels of vacuolization, necrosis, and immune cell infiltration at 24 h, there were significant differences in EROD activity induced by TCDF, PCB126, and TCDD. TCDF and PCB126 induced comparable EROD activity levels, resulting in ED_{50} values of 53.6 and 54.2 $\mu\text{g/kg}$, respectively. The EROD ED_{50} for TCDD was 1.1 $\mu\text{g/kg}$ comparable with the ED_{50} of 2.5 $\mu\text{g/kg}$ in B6 mice at 24 h (Ma *et al.*, 1992) and 1.6 $\mu\text{g/kg}$ in C57BL/6 mice at 7 days (Lin *et al.*, 1991). This is also in agreement with the TCDF EROD ED_{50} at 72 h in terms of maximum EROD induction as well as the ED_{50} values and REPs (Burgoon *et al.*, 2009). In addition, ED_{50} values for TCDD, TCDF, and PCB126 induction of Cyp1a1 messenger RNA (mRNA) levels (0.09, 2.5, and 3.9 $\mu\text{g/kg}$, respectively) were consistently lower compared with the corresponding EROD values (1.1, 53.6, and 54.2 $\mu\text{g/kg}$, respectively). The 0.09 $\mu\text{g/kg}$ TCDD Cyp1a1 mRNA ED_{50} from this study is almost identical to that reported by Abel *et al.* (1996) in C57BL/6 mice at 24 h (0.08 $\mu\text{g/kg}$). Others have also reported lower ED_{50} values for Cyp1a1 mRNA compared with EROD activity in B6C3F1 at the same time point (mRNA ED_{50} of 1.6 $\mu\text{g/kg}$ and EROD ED_{50} of 5.3 $\mu\text{g/kg}$) (Narasimhan *et al.*, 1994). Therefore, dose-dependent changes in gene expression may provide a more sensitive indicator of exposure, and possibly toxicity, provided an association can be established between gene function and the etiology of an adverse effect, assuming that a change in gene expression correlates with protein expression and activity.

TEF-adjusted equipotent doses of TCDD, TCDF, and PCB126 did not elicit comparable gene expression changes. TCDD elicited the greatest number of differentially expressed genes in comparison with TCDF and PCB126 at TEF-adjusted equipotent doses. Furthermore, TCDF- and PCB126-elicited changes in gene expression were a subset of the genes differentially expressed by TCDD. Suggestions of TCDF- and PCB126-specific gene expression responses have been previously shown to be a statistical artifact. When hard cutoff values ($P1(t) > 0.99$ and $|\text{fold change}| > 1.5$) are relaxed ($P1(t) > 0.90$ and $|\text{fold change}| > 1.5$), almost all PCB126- and TCDF-elicited genes become a subset of genes differentially

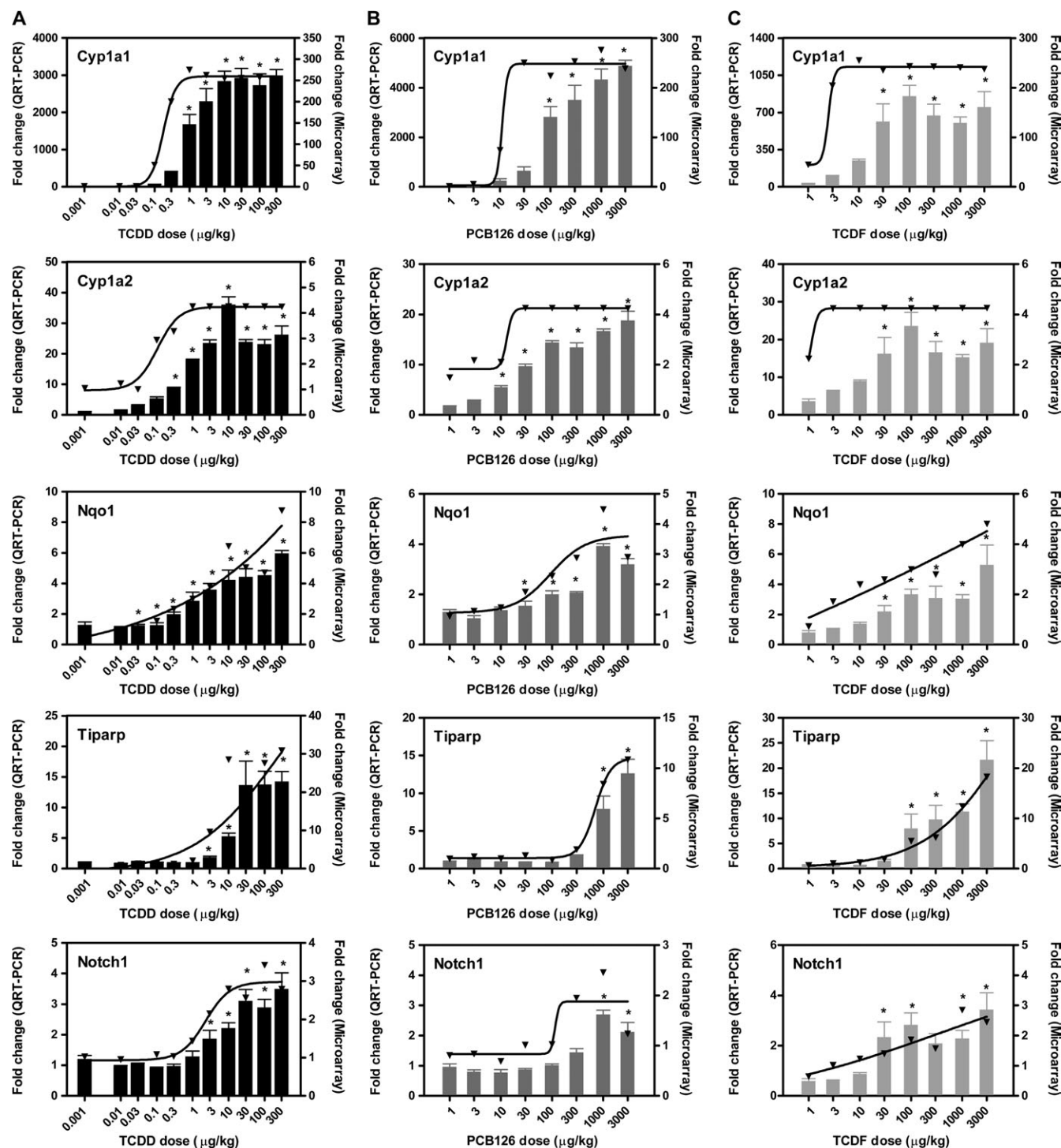


FIG. 7. QRT-PCR verification of selected AhR-regulated genes: Cyp1a1, Cyp1a2, Nqo1, Tiparp, and Notch1 for (A) TCDD, (B) PCB126, and (C) TCDF at 24 h. The same RNA samples used in the dose-response microarray studies were also used for QRT-PCR analysis. All fold changes were calculated relative to vehicle controls. Bars (left y-axis) and lines (right y-axis) represent QRT-PCR and microarray data, respectively. The genes are represented by their official gene symbols. Bars represent the mean \pm SE of four independent QRT-PCR samples. QRT-PCR data were analyzed by ANOVA followed by Dunnett's *post hoc* test: * $p < 0.05$ for vehicle versus treated samples. ED₅₀ values for microarray and QRT-PCR mRNA levels are provided in Supplementary table 7.

expressed by TCDD (Kopeck *et al.*, 2008; N'Jai *et al.*, 2008) (Fig. 6). However, the quantitatively lower number of TCDF- and PCB126-elicited differentially expressed genes cannot be

fully attributed to differences in ligand pharmacokinetics. HRGC/HRMS analysis indicates that the hepatic levels of TCDD, TCDF, and PCB126 at 24 h were essentially equipotent

when TEF adjusted. Consequently, the ability of the liganded AhR:ARNT complex to efficiently recruit the same complement of coactivators may also be an important factor for overall congener potency. For example, gene transactivation is reported to be congener structure-, coactivator-, and cell context dependent (Zhang *et al.*, 2008).

In order to calculate gene-specific REP values, TCDD-, TCDF-, and PCB126-elicited gene expression changes with a $PI(t) > 0.90$ and $|\text{fold change}| > 1.5$ were analyzed using our automated dose-response modeler (Burgoon and Zacharewski, 2008). REPs for only 210 TCDF versus TCDD genes and 40 PCB126 versus TCDD genes were calculated in order to comply with WHO guidelines requiring sigmoidal dose-response curves with similar slopes and shapes (Haws *et al.*, 2006; Van den Berg *et al.*, 2006). Overall, the TCDF median REP values at 24 h (0.06) and 72 h (0.1) are similar. This difference may be attributed, in part, to the use of a wider dose range and higher doses of TCDF and TCDD in the 24 h study and to the use of intact mice in the 72 h study. In contrast, the PCB126 REP of 0.02 reflects its weaker potency even at TEF-adjusted doses. PCB126 treatment also resulted in a lower number of differentially regulated genes exhibiting a sigmoidal dose-response when compared with TCDD. Many PCB126-elicited gene expression changes were excluded because of poor induction (< 1.5 -fold) or because they did not exhibit a sigmoidal dose-response curve. Alternatively, REPs based on points of departure (Burgoon and Zacharewski, 2008), lowest observable adverse effect level and/or no observable adverse effect level could be also considered (Van den Berg *et al.*, 2006).

In summary, this study expands the available hepatic REP data for TCDF and PCB126 using the immature ovariectomized C57BL/6 mice. It further suggests that at TEF-adjusted doses and at equipotent TEQ hepatic tissue levels, TCDF and PCB126 elicit weaker responses compared to TCDD. Therefore, in addition to pharmacokinetic differences, congener structure also plays an important role in gene expression potency and efficacy. Further associations of specific dose-responsive genes to toxic events are compromised by limited gene annotation and an incomplete understanding of the mechanisms involved in the etiology of the toxic responses elicited by TCDD and related compounds.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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